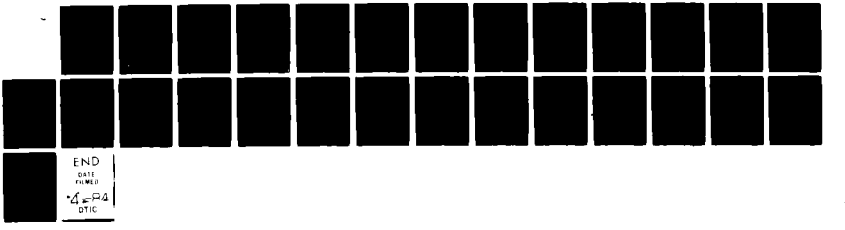
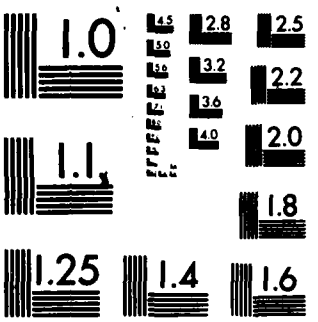
AD-A139 048 SAFE LIVE ORAL SALMONELLA VACCINES; USE OF ARO- STRAINS 1/1
(U) STANFORD UNIV CA DEPT OF MEDICAL MICROBIOLOGY
B A STOKER 20 MAY 83 DAMD17-79-C-9076

UNCLASSIFIED F/G 6/5 NL



END
DATE FILMED
4-84
DTIC



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AD_____

SAFE LIVE ORAL SALMONELLA VACCINES; USE OF *aro*⁻ STRAINS

ANNUAL REPORT AND FINAL REPORT

BRUCE A. D. STOCKER, M.D.

May 20, 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-79-C9076

Department of Medical Microbiology
Stanford University, Stanford, CA 94305

DOD DISTRIBUTION STATEMENT

A; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A139048 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) SAFE LIVE ORAL SALMONELLA VACCINES; USE OF aro⁻ STRAINS Final Report	5. TYPE OF REPORT & PERIOD COVERED Annual (2/8- - 8/81) Final (9/79 - 8/81) / 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Bruce A. D. Stocker	8. CONTRACT OR GRANT NUMBER(s) DAMD-17-79-C9076
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Medical Microbiology Stanford University Stanford, CA 94305	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102 A 3M161102 - BS01 TM 00 W 047
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE 5-20-83 / 13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) None / 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

A.
Distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Vaccines, bacterial live
Typhoid vaccines
Aromatic-deficient mutants
Salmonella sp., live vaccine
Mutants, aro⁻, as live vaccine
Salmonella typhi

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Tests of available reduced-virulence auxotrophic mutants of S. typhi showed the aspartate mutant to be ppc, deficient of phosphoenolpyruvate carboxylase; a PAB mutant to be affected at the locus mutated in S. typhimurium mutants pab-501 and pab-503 and mutant 479 to have an early block in purine biosynthesis, at an unidentified locus.

Complete blocks in aromatic or purine biosynthesis, or at ppc, resulting from insertion of transposon Tn10 were transduced into two wild-type S. typhi strains;

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

None

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

Figure 2

10 to the 4th power
10 to the 5th power

Block 20, Abstract (continued)

non-reverting secondary mutations were obtained. Defect at ppc did not much affect virulence, but the aro and pur strains were of greatly reduced virulence (LD50 in mouse, i.p., with hog gastric mucin, increased by 10^4-10^5) and an aro pur strain was even less virulent than either single-defect class. Such constructed strains, non-virulent but antigenically unaltered, may be of use as live, oral-route typhoid vaccines. S. typhi strains of low virulence because of pur defects may be of use as "safe" strains for laboratory teaching; aro strains, because unable to grow on media such as blood agar, are not suitable.

AD_____

SAFE LIVE ORAL SALMONELLA VACCINES; USE OF *aro*⁻ STRAINS

ANNUAL REPORT AND FINAL REPORT

BRUCE A. D. STOCKER, M.D.

May 20, 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-79-C9076

Department of Medical Microbiology
Stanford University, Stanford, CA 94305

DOD DISTRIBUTION STATEMENT

A; distribution unlimited

SUMMARY

Purpose. To test the effect of appropriate auxotrophic characters on virulence (in a small-animal model) of Salmonella typhi, and to construct and test strains appropriate for two possible applications. (i) Short term: provision of strains giving all the usual reactions of S. typhi but of much reduced virulence, therefore suitable for laboratory teaching, etc., as being unlikely to cause accidental infections. (ii) Long term: development of strains each with at least two non-reverting virulence-reducing auxotrophic characters, for possible use as oral-route live vaccines, for prevention of typhoid fever.

Methods. Previously reported reduced-virulence auxotrophic strains of S. typhi, so far as still available, were investigated. Transposon Tn10 insertions in relevant biosynthetic genes in S. typhimurium were transduced into S. typhi, and secondary mutations causing loss of tetracycline resistance and inability to revert to prototrophy were selected. Procedures for detecting even very infrequent reversion were developed. Virulence was tested by i.p. injection into mice, with hog gastric mucin.

Results. The available aspartate-responding low-virulent mutant was shown to be of class ppc, deficient of phosphoenolpyruvate carboxylase. S. typhimurium transposon mutants received as asp::Tn10 were found to have the same defect. This defect transduced into S. typhi did not cause any large increase in LD50 in the mouse model. The PAB-responding mutant was confirmed as such, and shown to be mutated at (or close to) the locus mutated in two pab mutants in S. typhimurium. Mutant 479 was shown to have an early block in purine biosynthesis but the locus affected was not identified. The purine-requiring mutant of the "Panama carrier" strain was confirmed as such--but both it and mutant 479 reverted to purine independence at readily detectable rates (as also did the pab mutant).

Tests of recent isolates of S. typhi showed that some strains could grow without tryptophan, others only if given tryptophan, and two strains if given either indole or tryptophan; some strains had requirements for growth factors other than tryptophan or cystine.

Strains with non-leaky, non-reverting defects in aromatic biosynthesis and/or in purine biosynthesis were derived from two wild-type S. typhi strains, Ty2 (a well-characterized strain of phage type E1, and parent of the galE live-vaccine strain Ty21a of Germanier) and CDC10-80 (a recent isolate of phage type A, expected to be more amenable to transduction of genes from S. typhimurium because of its non-restricting phage type). The defects at aroA or at various pur loci were introduced either by transduction of Tn10 insertion mutations from S. typhimurium, with selection for tetracycline-resistance, or, in some instances, by co-transduction of an aroA deletion mutation (previously characterized in S. typhimurium) with an adjacent silent Tn10 insertion, by selection for tetracycline resistance. Mutations causing loss of tetracycline resistance were selected from Tn10 insertion transductants and screened for inability to revert to aromatic independence or purine independence. Both pur and aroA defects caused very considerable loss of virulence in the mouse model. A derivative of strain Ty2 with non-reverting aroA and pur defects had an LD50 value in mice ca. 10^7 greater than that of the wild-type parent strain. Strains with aroA defects did not grow well on various media, including blood agar, unless these were supplemented with 2,3-dihydroxybenzoic acid, to allow synthesis of the iron-capturing compound enterochelin or enterobactin; they are thus not suitable as "teaching strains."

Conclusions. The feasibility of constructing strains of S. typhi with two independent non-reverting virulence-reducing biosynthetic defects has been proven; such strains may prove appropriate as "candidate" oral-route live vaccines, for prevention of typhoid fever. Strains of S. typhi appropriate for use in laboratory teaching, because of much reduced virulence but giving typical reactions, have been prepared.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publ.No (NIH) 78-23,Rev. 1978).

TABLE OF CONTENTS

Army Contract, Final Report

Introduction. The objective of the project was to examine the effect of various auxotrophic characters on the virulence of Salmonella typhi, using appropriate genetic techniques to construct strains with the desired character or characters, both because of the theoretical interest of this subject and because of two practical applications: First, in the short term, the construction of a strain or strains of S. typhi typical of the species in all characters used in the diagnostic laboratory (antigens, biochemical characters, phage sensitivity, etc.) but non-virulent because of auxotrophy, therefore suitable as a strain for laboratory teaching, proficiency testing, source of Vi or other antigens, etc., as being unlikely to cause accidental infection of laboratory workers. Second (long term) the construction and testing of a strain or strains of S. typhi of unaltered antigenic character but having at least two non-"leaky," non-reverting auxotrophic characters, each by itself causing substantially complete loss of virulence, as the first stage in production of a safe and effective live-vaccine strain for oral-route immunization in man. The results achieved in the two-year funded period (and extension without additional funds for 12 months) are summarized below.

Investigation of previously reported auxotrophic defects causing reduction of virulence in S. typhi. The first substantial work on effect of auxotrophy on virulence in Salmonella typhi was the investigation of Bacon, Burrows and Yates(1950a,b; 1951) at Porton, England, on the virulence (in the mouse, intraperitoneal route in saline, model) of many X-ray-induced auxotrophic mutants of a prototrophic derivative, Ty22, of strain Ty2. This work showed that mutants with requirements for either a purine (or a purine + thiamine), or for aspartate, or for, in one mutant, p-aminobenzoate, were of reduced virulence, as shown by an increase in the LD50 dose in the mouse model infection used. The authorities at Porton sent us the only available representatives, one mutant of each of these three classes (the other mutants having been discarded some time back). All three strains were recovered from the dried material supplied. Strain 479, reisolated from a single colony on

rich medium, was confirmed as requiring either adenine or guanine (and was labeled 208Ty in our collection); the accumulation of pigment during growth on defined medium indicated that the block in purine biosynthesis in this strain is at a late step but attempts to identify the pur locus concerned were unsuccessfull, since transduction tests with pur mutants of S. typhimurium as donors gave only equivocal results. This mutant reverted to purine-independence at an easily detectable rate. Since methods for obtaining non-reverting defects on purine biosynthesis were devised (see below) mutant 479 (= 208Ty) was not further investigated.

Mutant 8505 after single-colony reisolation was confirmed as auxotrophic, responding to aspartate (and was assigned number 210Ty in our collection). Since the only specific step in the biosynthesis of aspartic acid is transamination, for its production from oxaloacetate and since three different transaminases are expected to be able to effect this reaction, it was a matter of some surprise that a single mutation should result in aspartate auxotrophy. Investigation of this phenomenon was facilitated by the receipt from Dr. John Roth (Department of Biology, University of Utah) of several aspartate-responding auxotrophic derivatives of S. typhimurium strain LT2 obtained by transposon mutagenesis, i.e., with the tetracycline-resistance transposon Tn10 inserted in the affected gene. We thought that if aspartate requirement did regularly produce a substantial loss of virulence, we would be able to introduce this character into any desired S. typhi strain by transduction from one of the mutants supplied by Dr. Roth, by selection for tetracycline-resistant transductants. However, to our surprise, all the strains supplied as asp::Tn10 grew well on our simple defined medium, not supplemented with any amino acid. The explanation for the apparent discrepancy between our results and those of Roth proved to be a difference in the defined medium used as base for tests of nutritional character: We used agar of the Davis-Mingioli formula, with glycerol, 5 ml/l, as energy source but with sodium citrate, 0.44 g/l, included to prevent precipitation of cations as phosphate, etc., whereas the medium used in Dr Roth's laboratory contained glucose as energy source and no citrate. S. typhimurium can assimilate citrate and use it as sole carbon source, whereas S. typhi is "citrate-negative" indicating that it cannot grow on (unsupplemented) defined medium with citrate as sole energy source. Further investigation showed that all the "asp::Tn10" strains received would grow if a glucose-based simple

defined medium were supplemented either with aspartate or with citrate or with various other Krebs-cycle intermediates. A representative mutant of this class, and also the S. typhi aspartate-responding mutant,8505, of Bacon et al.(1950a,b), were found to be defective of phosphoenolpyruvate carboxylase enzymic activity, i.e., in ability to condense phphoenolpyruvate with carbon dioxide to form α-keto-glutarate, a reaction required for resupplying 4-carbon skeletons for the Krebs cycle, to replace those withdrawn for synthesis of aspartate and several amino acids, purines, etc. Strains of S. typhimurium lacking this enzyme can utilize either aspartate or citrate, or other Krebs cycle intermediates, for this purpose, whereas the S. typhi mutant with the same defect cannot utilize citrate (presumably because of defective uptake) but can make use of aspartate, to restock the Krebs cycle. This discovery explains the observation of Bacon et al.,(1950a,b) that the amount of aspartate needed to obtain satisfactory growth of mutant 8505 (and of other aspartate-responding mutants, no longer available for test but presumably with the same enzymic defect) was much greater than the amount of relevant amino acid required to obtain good growth of amino acid auxotrophs of other classes (because in S. typhi lacking phosphoenolpyruvate carboxylase aspartate must serve as precursor of several aminoacids and of purines,etc, as well as of aspartate in protein).

Strain 20063 after single-colony reisolation was confirmed as requiring p-aminobenzoate (and was labeled 203Ty in our collection). Genetic analysis showed that its mutation (assigned allele number pab-41) was closely linked to those of two p-aminobenzoate-requiring mutants in S. typhimurium LT2, pab-501 and pab-503. The locus in question has not yet been identified, as corresponding to pabA or pabB of E. coli, or as representing some other locus. Strain 20063 reverted to prototrophy at a fairly low rate. However,since no method of procuring non-reverting pab mutations is available this strain was used as parent of double-auxotroph derivatives, as described below.

Strain 41-A-58, originally described as a non-virulent (for mouse) spontaneous variant isolated from the "Panama carrier" and later identified as a purine auxotroph, was received from Dr. L. Baron as WR4023. A single-colony reisolate was confirmed as a purine auxotroph. However, this strain was found to revert to purine independence at high frequency and it was therefore not

further investigated.

Nutritional Character of Wild-type Salmonella typhi. It is well-known that at least some strains of Salmonella typhi will not grow on chemically defined media which do not contain tryptophan. To test the validity and generality of this, a collection of recently isolated strains, received from the Center for Disease Control, Atlanta, or from the California State Public Health Laboratory, Berkeley, were tested for ability to grow on a semi-defined medium, composed of a simple defined medium supplemented with a small amount of acid-hydrolyzed (therefore tryptophan-free) casein and cystine. Nine strains grew on this medium only if it was supplemented with tryptophan, and not if supplemented, instead, with indole or anthranilic acid. Two strains grew if the medium was supplemented either with tryptopha or with indole, but not if supplemented, instead, with anthranilic acid. Ni strains grew on the test medium even if no supplement was added, and thus ar ble to synthesize their own supply of tryptophan. Eight strains failed to even on the tryptophan-supplemented medium, because of nutritional requirements for metabolites other than any of the common amino acids.

Construction of Auxotrophic S. typhi Strains. The main genetic methods used for constructing strains with virulence-reducing auxotrophic characters were: (i) transduction of Tn10-insertion mutations either in, or adjacent to, relevant biosynthetic genes, into S. typhi, by application of phage P22 HT105/1 int lysates of S. typhimurium donor strains, and selection for tetracycline-resistant transductants; (ii) isolation of Tn10-generated deletion or deletion-inversion mutants of S. typhi strains having Tn10 insertions in relevant biosynthesis genes, by selection for tetracycline-sensitivity on the medium described by Bochner et al.,(1980); (iii) testing of Bochner-selected tetracycline-sensitive mutants to see if they could regain the relevant biosynthetic function by reversion, even at very low frequency. Transductants acquiring Tn10 insertion mutations were selected by the procedure previously used with S. typhimurium as recipient, except that phage lysates, usually of titers between 2×10^9 and 10^{10} p.f.u./ml., were used undiluted, because the yield of tetracycline-resistant transductants in the crosses with S. typhi as recipient were much lower, usually 10^{-2}-10^{-3}-fold, than when the recipient was S. typhimurium. Two strains of wild-type character were chosen as parent strains of auxotrophic derivatives; in

addition, second auxotrophic characters were introduced into two of the auxotrophic strains from the Porton investigation. S. typhi strain Ty2 (received from Dr. L. Baron as WR4014) was chosen as one parent strain, because it is well characterized, of relatively high mouse-virulence and is the parent of the proven effective galE live-vaccine strain Ty21a, of Germanier. However, strain Ty2 is of phage type E1, known to have a strong restricting effect (on Vi phage II not having the appropriate DNA modification), which was expected to reduce the frequency of transduction of genes from S. typhimurium, since these would not have the appropriate DNA modification. A recently isolated strain of the non-restricting phage type A, received from the CDC as CDC10-80, and found to give all the usual reactions of S. typhi, was chosen as the second parent strain, expected to give significantly higher yields of transductants than strain Ty2. Selection of tetracycline-sensitive variants from Tn10-bearing strains was effected by selection on Bochner medium with a modification previously found necessary in the case of S. typhimurium strains with aro defects, i.e., supplementation with 2,3-dihydroxybenzoic acid. This addition allows such strains to manufacture the iron-capturing compound enterobactin or enterochelin, despite their defect in aromatic synthesis; aro strains, even if tetracycline-sensitive, failed to grow on Bochner medium not so supplemented. Tests for ability of auxotrophic (aro or pur, etc.) strains to revert to aromatic independence, etc, were made by the method previously used for testing live-vaccine strains of S. typhimurium (Smith et al.,1983). The test medium used was a defined-medium agar supplemented with an acid hydrolysate of vitamin-free casein (to supply amino acids other than tryptophane) and a more than sufficient supply of any other requirements of the strain used, except one: either tryptophan, in the case of aro mutants, or adenine, for pur mutants. A growth-limiting amount of one of these compounds was added, either as the pure substance alone, or as a known amount of the pure substance together with "Oxoid" nutrient broth, CM67, 1% by volume. The content of the growth-limiting supplement was such as to allow growth of the auxotrophic strain to a final population of about 10^{10} colony-forming units per plate, forming a thin film of growth. Efficiency of detection of revertants was investigated by adding a few aro^+ or pur^+ but otherwise isogenic bacteria at one point on a plate already incubated for two or more days; the development of a corresponding number of colonies on further incubation showed that even a

single revertant amongst the 10^{10} auxotrophic bacteria on a plate would have been detected by its growth into a visible colony.

The main strains constructed, with their origin and relevant characters, are listed in Table 1, which includes the results of LD50 determinations in mice, made on certain strains, as described below. Strains of particular interest, either as possible "safe" strains for laboratory teaching, or as "model" live-vaccine strains, or otherwise, are discussed individually, in a later section.

Tests of mouse virulence. We needed to find an infection model in which the LD50 of wild-type S. typhi in mice would be small, so that we could test the non-lethality of live inocula of constructed strains given in doses several log units higher than the LD50 of the wild-type strains. The i.p. route, hog gastric mucin, 5%, supplement, described in the "official" test of immunizing efficiency of killed S. typhi vaccines, sounded suitable, since the LD50 dose of S. typhi wild-type in non-vaccinated mice in this test is required to be <10. In practice, we could not achieve such low LD50 values even for strain Ty2 (and we found, on inquiry to laboratories concerned with testing S. typhi vaccines, that this difficulty has been encountered elsewhere). After trial of several mouse lines, and of several lots of hog gastric mucin, reasonably satisfactory results were achieved by use of one combination of these two factors, and strain Ty2 or CDC10-80. The batch of hog gastric mucin used, kindly supplied by a colleague in Sweden who had obtained satisfactory results with it, had an iron iron content, determined by atomic adsorption, of 1.85 mg/g, which is within the range found typical of lots of hog gastric mucin giving satisfactory results in this test by Powell and his colleagues (1980). Even with this material the LD50 values obtained in mice of several lines were greater than 1,000; however, in outbred mice of stock CFW(SW)BR obtained from Charles River Breeding Labs the approximate LD50 values found were, for strain Ty2 wild-type, 20, and for strain CDC10-80, wild-type, 500. (Note that exact LD50 values were not essential for our purpose and that, for economy, only small numbers of mice were used, but sufficient for detection of large alterations of LD50 value, such as we were looking for.) The LD50 values for several constructed strains which were tested in this respect are recorded in Table 1, and those for strains of particular interest are considered below.

Biochemical and Other in vitro Tests of Constructed Strains. Transductants and Bochner-selected mutants were routinely tested with anti-Vi serum, to confirm their id. rity as S. typhi, rather than contaminant. The introduction of an rpsL allele conferring high-level streptomycin-resistance into parent strain CDC10-80, to give strain 215Ty, did not cause any other evident change in in vitro characters. Introduction of pur::Tn10 and ppc::Tn10 by transduction caused the expected new auxotrophic characters, but no other evident alteration in biochemical, serological, etc., characters, in either of the two lines. The rate of growth of the strains with these new auxotrophies, as judged by colony size either on rich medium or on appropriately supplemented defined medium, was not obviously different from that of the parent strains. The situation was different for the aroA554::Tn10 transductants, and their tetracycline-sensitive derivatives. All such S. typhi strains grew somewhat slowly and irregularly, with very heterogeneous colony size, on "rich" media, such as Oxoid nutrient agar, CM55, and failed to grow on some media supplemented with blood or serum. This behavior evidently resulted from their inability to acquire iron efficiently, since supplementation of any of these media with dihydroxybenzoate restored normal growth. The results of biochemical, etc., tests in the API20E system, obtained with constructed strains of possible utility, are described below.

Constructed Auxotrophic S. typhi Strains of Possible Utility. Two tetracycline-sensitive, non-reverting (to purine independence) mutants, 247Ty and 248Ty, were obtained by Bochner selection from Ty2 made pur-1735::Tn10 (Table 1). These two strains retained all the biochemical characters of their wild-type ancestor and, like it, gave code 2004540 in the API 20E test. This score differs from the "modal" S. typhi API 20E code, 4004540, because strain Ty2 scored + on the LDH test, whereas 72% of S. typhi strains in the API data bank were negative, and (usually) negative in the LDC test, recorded as positive for 99% of S. typhi strains in the data bank. These two strains, and strain Ty2, their wild-type precursor, were, by courtesy of Dr. Marjorie Bissett, examined by the State of California Department of Health Sciences, Microbial Diseases Laboratory, at Berkeley; all three were reported as S. typhi phage type E_1, with no anomalous behavior noted in the range of fermentation and other tests routinely used for strains suspected to be S. typhi. One of these two stable purine-requiring strains, 247Ty, has been

tested for mouse virulence, by the technique described above. In the first test all of five mice given 60,000 colony-forming units (the largest dose tested) survived, whereas in a simultaneous test the LD50 of strain Ty2 was ca. 600; in a repeat test 3 of 8 mice survived an inoculum of 60,000,000 of 247Ty, while only 4 of 8 mice survived 180 colony-forming units of Ty2. Though the number of mice used was not sufficient for precise LD50 estimations, it appears that the purine auxotrophy of strain 247Ty has caused an increase in LD50 value of at least five log-10 units. No reversion to purine independence has been detected either in in vitro tests of 247Ty or by culture from mice which died from the 60,000,000 challenge dose. More extensive testing of the nature of the pur genetic lesion in strain 247Ty would be desirable, to further confirm its inability to revert, and evidently non-virulence for man cannot with certainty be predicted from the results of mouse experiments; with these limitations, however, it seems that strain 247Ty should be satisfactory as a presumptively "safe" strain of S. typhi for laboratory teaching and the like. We do not propose it as a candidate live-vaccine strain, because it owes its non-virulence to a single genetic defect. This defect might, in theory at least, be corrected by a single recombinational event, transduction or conjugation, in the intestinal tract of a subject given the strain by mouth.

Strain 251Ty is a tetracycline-sensitive mutant of Ty2 made aroA554::Tn10 by transduction. It was found to revert to aromatic-independence in vitro at a low frequency and to accumulate revertants during storage at room temperature. However, it was tested in mice before its ability to revert had been detected. Three of six mice given 2,400,000 of this strain survived, but aromatic-independent S. typhi were recovered at autopsy of one of the mice which succumbed. Strain 251Ty has been discarded, because of its ability to revert; however, it is noteworthy that despite this ability it was of reduced virulence, with an LD50 value more than three log-10 units greater than that of strain Ty2, tested at the same time.

Strain 262Ty is a tetracycline-sensitive mutant of strain Ty2 made zbj-903::Tn10 CRR401[aroA554::Tn10(Tc-sens,non-rev)] by transduction. Its CRR (complex rearrangement) mutation, probably a deletion-inversion, introduced by co-transduction with the silent Tn10 insertion, came from an S. typhimurium strain, SL3235, which has been extensively tested both for in vitro reversion

to aromatic independence, and for non-virulence, both in mice and (after it was made streptomycin-resistant) in calves (Hoiseth & Stocker, 1981; Hoiseth, Ph.D. thesis, Stanford University, 1982; Smith et al., 1983). Because strain 262Ty (like other aro⁻ strains) cannot grow on media such as blood agar it is not appropriate a a safe teaching strain. It would be expected to be an effective live vaccine, but would not be as safe as a strain with two independent virulence-reducing defects.

Strain 253Ty was constructed from strain 247Ty, the non-virulent purine auxotrophic descendant of Ty2, described above, by introduction of a second virulence-reducing auxotrophy. Mutation aroA554::Tn10 was first transduced into strain 247Ty, then a tetracycline-sensitive derivative, found non-reverting (to aromatic independence), was obtained by selection on Bochner medium. In the only mouse test made of strain 253Ty its LD50 value was ca. 1,000,000,000, i.e., close to the toxic LD50 value. Thus the presence of both an aroA and a pur block caused loss of virulence greater than that caused by either single defect by itself, as measured by LD50 value in the mouse test used.

Most of the strains derived from the second wild-type parent, CDC10-80, of phage type A, were made from its streptomycin-resistant transductant, strain 215Ty. Mutation to streptomycin-resistance, presumably by mutation at locus rpsL, is known to cause substantial loss of virulence in at least some Shigella sp. strains; however, the introduction of rpsL107 into strain 215Ty did not cause any substantial loss of mouse virulence, since in two tests of strain 215Ty its LD50 values were <1,000 and ca. 500.

Strain 235Ty, a non-reverting, tetracycline-sensitive mutant of a pur-1735::Tn10 transductional derivative of 215Ty, behaved in vitro as a typical S. typhi strain. In two tests in mice it gave LD50 values of ca. 30,000,000. This strain (like the stable pur derivatives, 247Ty and 248Ty, in the Ty2 line) may be appropriate for use as a teaching strain, but it has not yet been investigated for phage type and some biochemical characters.

Strain 271Ty is CDC10-80 made streptomycin-resistant, then zhj-903::Tn10 CRR401,etc, by the same procedure as described above, for the corresponding strain in the Ty2 lineage. It thus has an aro defect already extensively tested in S. typhimurium for non-reversion and virulence-reducing effect. The LD50 value obtained in a single test of strain 271Ty was about 20,000,000. A

tetracycline-sensitive mutant of 271Ty was isolated, as strain 272Ty, for possible use as recipient in further transductional steps. This derivative has not yet been tested for virulence.

Strain 234Ty is a tetracycline-sensitive mutant of a ppc-546::Tn10 transductant isolated from the streptomycin-resistant version of CDC10-80. It thus has the same biosynthetic defect as the only available aspartate-requiring mutant, of reduced virulence, from those described by Bacon, Burrows and Yates (1950a,b). However, in the only mouse-virulence test made, none of three mice given 12,000 colony-forming units (smallest dose tested) of strain 234Ty survived. Thus, the loss of virulence for mutants of this class observed by Bacon and his colleagues was not found in our constructed strain. It is not clear whether this difference is a consequence of different genetic background, CDC10-80 instead of Ty2, or of the different mouse system used, with hog gastric mucin or in saline, etc., or of some other cause.

Effect of auxotrophy on virulence in Salmonella typhimurium. Work on the effect of non-leaky non-reverting mutations in the aromatic biosynthetic pathway on the mouse-virulence of S. typhimurium and S. dublin has been undertaken in Dr. Stocker's laboratory with support mainly from other sources (NSF Fellowship for Susan Hoiseth; NIH support to Dr. Stocker, for part of the period of the contract and from SmithKline Animal Health Products, for one aspect of the work, for part of the period), but with some contribution from the DAMD contract. The main conclusions from this work are reported in Hoiseth & Stocker, 1981, Nature, 291:238-239 (see Appendix A). In addition the effect of a block in purine biosynthesis caused by insertion mutation pur-1735::Tn10 on mouse virulence of S. typhimurium was investigated by Ron Brown, because this was the purine biosynthesis defect most used to produce non-virulence in S. typhi. The S. typhimurium strain used as recipient was SL1344, a genetically marked (hisG46) derivative of a "wild" strain, proven calf-virulent and mouse-virulent, and itself proven calf-virulent by calf passage (by a collaborator, Dr. Bradford Smith, in the Department of Medicine, School of Veterinary Medicine, University of California at Davis). The pur-1735::Tn10 derivative of SL1344 was designated 144RB. Strain SL1344 had an LD50 value, in BALB/c mice, inoculated i.p., of <150 colony-forming units; only one of 5 mice survived injection of 150 bacteria, to day 12. The LD50 of its purine-requiring derivative, 144RB, was $>1.5 \times 10^6$ (no deaths, to day 12,

in groups of 5 mice given 150 or 1.4×10^4 c.f.u., one death amongst 5 mice given 1.5×10^6 c.f.u.). On day 12 the survivors in the three groups given strain SL1344, together with 8 non-vaccinated mice of the same age, were challenged by intraperitoneal injection of 2.7×10^4 bacteria of the virulent strain, SL1344 (i.e., more than 100 LD50 doses). All the non-vaccinated control mice died, by day 5. All the live-vaccinated mice, even those which had received only 150 bacteria of strain 144RB, survived, to day 11 after challenge. This result suggests that the same biosynthetic defect introduced into wild-type S. typhi, for use as a live vaccine, is likely to cause a satisfactory loss of virulence without loss of immunizing ability.

Discussion and conclusions. The successful construction of S. typhi strains with transposon-generated non-leaky non-reverting biosynthetic blocks in aromatic biosynthesis and/or in purine biosynthesis established the feasability of the method proposed for construction of non-virulent but antigenically unaltered strains of S. typhi, for use as oral-route live vaccines. The great reductions in (mouse) virulence, as tested by i.p. injection, with hog gastric mucin adjuvant, i.e., several log units increase in LD50, shows that the defects in aromatic biosynthesis or in de novo purine biosynthesis greatly reduced virulence in S. typhi. This was as had been predicted from earlier observations on effect of such defects on virulence of S. typhimurium and some other pathogenic bacteria, both in Dr. Stocker's laboratory and elsewhere. (By contrast transposon-generated complete defects in phosphoenolpyruvate carboxylase, causing aspartate requirement, did not cause any large increase in LD50, contrary to expectation from the reduced virulence, in a somewhat different animal model (mouse, i.p. route, but large dose, without adjuvant) of aspartate auxotrophs in S. typhi reported by Bacon, Burrows and Yates (1950a,b). The one available aspartate auxotroph, of reduced virulence, from their investigation was shown to owe its auxotrophy to this enzymic defect. The combination of an aro and a pur defect in a single S. typhi strain has been shown to cause a greater loss of virulence, as measured by increase in LD50 value in the animal model used, than either defect alone. These results establish the feasibility of construction of candidate strains, for trial as oral-route vaccines in man. Such vaccines, if found safe and effective, would have obvious advantages over the commonly used killed vaccine, as already observed for the Germanier galE vaccine in the field trial in Egypt. S. typhi made non-virulent by appropriate auxotrophic

defects may prove to have some advantages over the galE vaccine: (i) Greater guaranty of non-reversion, because of more complete knowledge of the nature of the genetic defects and because of presence of two independent non-reverting virulence-reducing genetic lesions; (ii) Greater guaranty of safety, even in immunodeficient recipients, because non-virulence does not depend on host defense mechanisms; (iii) Possibly greater immunizing efficiency, in that a single oral dose may prove to be effective, as suggested by results in oral-route immunization of mice with constructed S. typhimurium live-vaccine strains(Hoiseth and Stocker,1980, see Appendix A).

A short-term objective of the project was the production of S. typhi strain(s) which would give all the ordinary reactions of S. typhi observed in the diagnostic microbiology laboratory, but be non-virulent or, at least, of greatly reduced virulence. Such strains could be used with no, or very little, risk of laboratory infection, both for teaching purposes, for proficiency tests and the like, for mass growth for production of Vi antigen, etc., and in some research projects. S. typhi strains given aro defects proved not to be suitable for this purpose, because their inability to make enterobactin (≈ enterochelin), unless supplied with dihydroxybenzoate, makes them unable to grow on some commonly used media, such as blood agar, because of their inability to capture iron from transferrin, etc. Strains with Tn10-generated blocks in purine biosynthesis, so far as tested at the time of termination of support, appeared to have the desired combination of characters. Furthermore the p-aminobenozoate auxotroph described by Bacon, Burrows and Yates (1950a,b) would itself, probably, be of reduced virulence for man, yet typical in behavior in vitro; however, this strain was found to revert to p-amino-benzoate-independence (and so, presumably, to normal virulence) at an easily detectable rate. A constructed derivative of this strain, with a Tn10-generated pur defect, non-reverting, should, however, be satisfactorily safe; so far as tested it appeared satisfactory also in respect of behavior in common diagnostic tests.

Recommendations. The project begun with support of the contract, i.e., the construction of a safe and efficient oral-route live-vaccine strain for prevention of typhoid fever, has been resumed in Dr. Stocker's laboratory, with support from SmithKline-RIT, Belgium. It seems likely that construction

of a strain with properties justifying trial as oral-route vaccine in human volunteers will soon be completed. If such a strain gives satisfactory results, in respect of inocuity and ability to confer protection against challenge in volunteers, the Army Medical Service (and other Uniformed Services) should, it is suggested, consider adoption of a live, oral-route vaccine, to replace killed vaccine for immunization of service personnel. [A recently reported death of a healthy adult, apparently resulting from administration of killed typhoid vaccine (The Daily Californian, March 29, 1983, pp. 1 and 6, see Appendix B) and earlier reports of severe or even fatal reactions (Wilson, 1967) indeed call in question the advisability of continued administration of killed S. typhi vaccines to persons not at special risk.]

The constructed strains of S. typhi, if confirmed non-virulent by available animal tests, yet typical in behavior in ordinary laboratory tests, should be adopted as "teaching strains" so far as such are needed for laboratory courses, proficiency testing and the like in U.S. Army medical establishments.

Table 1. *Salmonella typhi* starting and constructed strains.

Strain no.	Constitution	How obtained	LD50
(a) Ty2 lineage			
200Ty (= Ty2)	Ty2 wild-type	From L. Baron as WR4014	ca.200
240Ty	Ty2 *pur-1735*::Tn10	From 200Ty by transduction	n.t.
247Ty	Ty2 CRR467[*pur-1735* (Tc-sens,non-rev)]	From 240Ty by Bochner selection	ca.6×10^7
248Ty	Ty2 CRR468[*pur-1735*::Tn10 (Tc-sens)]	From 240Ty by Bochner selection	n.t.
250Ty	Ty2 *aroA554*::Tn10	From 200Ty by transduction	n.t.
251Ty*	Ty2 CRR469[*aroA-554* (Tc-sens)]	From 250Ty by modified Bochner selection	ca.2×10^6*
261Ty	Ty2 *zbj-903*::Tn10 CRR401[*aroA554*::Tn10 (Tc-sens,non-rev)]	From 200Ty by co-transduction	ca.10^7
262Ty	Ty2 CRR471[*zbj-903*::Tn10 (Tc-sens)] CRR401[*aroA554*::Tn10(Tc-sens,non-rev)]	From 261Ty by modified Bochner selection	n.t.
252Ty	Ty2 CRR467[*pur-1735*::Tn10 (Tc-sens,non-rev)] *aroA554*::Tn10	From 247Ty by transduction	n.t.
253Ty	Ty2 CRR467[*pur-1735*::Tn10 (Tc-sens,non-rev)] CRR570 [*aroA554*::Tn10 (Tc-sens, non-rev)]	From 252Ty by modified Bochner selection	ca.109

Strain no.	Constitution	How obtained	LD50
(b) CDC10-80 lineage			
211Ty	CDC10-80 wild-type	From C.D.C.	n.t.
215Ty	CDC10-80 rpsL107	From 211Ty by transduction	ca.500
217Ty	CDC10-80 rpsL107 pur-1735::Tn10	From 215Ty by transduction	n.t.
235Ty	CDC10-80 rpsL107 CRR559 [pur-1735::Tn10 (Tc-sens)]	From 217Ty by Bochner selection	ca.3×10^7
271Ty	CDC10-80 rpsL107 zbj-903::Tn10 CRR401[aroA554::Tn10 (Tc-sens,non-rev)]	From 215Ty by co-transduction	ca.2×10^7
272Ty	CDC10-80 rpsL107 CRR478 [zbj-903::Tn10(Tc-sens)] CRR401[aroA554::Tn10 (Tc-sens,non-rev)]	From 271Ty by modified Bochner selection	n.t.
233Ty	CDC10-80 rpsL107 ppc-546::Tn10	From 215Ty by transduction	n.t.
234Ty	CDC10-80 rpsL107 CRR478[ppc-546::Tn10 (Tc-sens)]	From 234Ty by Bochner selection	<25,000

* Mutation CRR471, causing loss of tetracycline resistance in strain 251Ty, did not prevent reversion to Aro$^+$ at low frequency *in vitro*, and Aro$^+$ bacteria were recovered autopsy from a mouse which died after i.p. inoculation with strain 251Ty, with hog gastric mucin.

LITERATURE CITED

Bacon, G.A., Burrows, T.W. and Yates, M. (1950a). The effects of biochemical mutation on the virulence of Bacterium typhosum: The induction and isolation of mutants. Br. J. exp. Path. 31:703-713.

Bacon, G.A., Burrows, T.W. and Yates M. (1950b). The effects of biochemical mutation on the virulence of Bacterium typhosum: The virulence of mutants. Br. J. exp. Path. 31:714-724.

Bacon, G.A., Burrows, T.W. and Yates, M. (1951). The effects of biochemical mutation on the virulence of Bacterium typhosum: The loss of virulence of certain mutants. Br. J. exp. Path. 32:85-96.

Hoiseth, S.K and Stocker, B.A.D. (1981). Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. Nature, Lond. 291:238-239. (= Appendix A in this report.)

Powell, C.J. DeSett, C.R. Lowenthal, J.P. and Berman, S. (1980). The effect of adding iron on the enhancement of virulence for mice of Salmonella typhi strain TY2. J. Biol. Stand. 8:79-85.

Smith, B.P., Reina-Guerra, M., Hoiseth, M.K., Stocker, B.A.D., Habasha, F., Johnson, F. and Merritt, F. (1983). Aromatic-dependent Salmonella typhimurium as modified live vaccine for calves. Amer. J. Vet. Res. (in press).

Wilson, Sir G.S. (1967). pp 250-252, in The Hazards of Immunization. University of London Athlone Press, London.

Reprinted from Nature, Vol. 291, No. 5812, pp. 238-239, May 21 1981

Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines

Susan K. Hoiseth & B. A. D. Stocker

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305, USA

An auxotrophic mutant which requires a metabolite not available in vertebrate tissues should be unable to grow in such tissue and thus be non-virulent. Most mutations to auxotrophy do not affect virulence[1-3], presumably because the required metabolites are available at sufficient concentration. However, Bacon *et al.*[1,2] noted that a *Salmonella typhi* mutant requiring *p*-aminobenzoic acid (*p*AB) was less virulent (for mice) than its parent. *Salmonella*, unlike vertebrates, cannot assimilate exogenous folate and must synthesize it from *p*AB; the virtual absence of *p*AB from vertebrate tissues is shown by the efficacy of sulphonamide chemotherapy. Yancey *et al.*[4] reported reduced virulence for a *S. typhimurium* mutant with a requirement for 2,3-dihydroxybenzoate (DHB), the precursor of the bacterial iron-acquisition compound, enterochelin. As DHB is not a known vertebrate metabolite, it would be expected to be absent from mouse tissues. *Salmonella* synthesize *p*AB and DHB from chorismate, the final product of the aromatic biosynthetic (*aro*) pathway. A complete block at any step of this pathway should make *S. typhimurium* auxotrophic for two compounds not available in vertebrate tissues, and thus non-virulent. We describe here the use of a tetracycline-resistance transposon, Tn*10* (refs 5, 6), inserted in gene *aroA* to produce non-reverting, aromatic-requiring derivatives of virulent *S. typhimurium* strains. These derivatives were virtually non-virulent; their use as live vaccines conferred excellent protection against challenge with a virulent strain.

The *aro* transposon insertion used was *aroA554*::Tn*10*, in the chromosome of *S. typhimurium* strain LT2. As wild-type LT2 is only weakly pathogenic we transduced the *aroA554*::Tn*10* region into a virulent *S. typhimurium* strain, using phage P22 HT105/1 *int* (ref. 7) and selecting for tetracycline resistance. The virulent strain used as recipient, SL3201, is a mouse-passaged re-isolate of the genetically marked mouse-virulent *S. typhimurium* strain SL4522, of biotype FIRN[8]. Two of the transductants obtained, SL3217 and SL3218, required tryptophan, tyrosine and phenylalanine. Their additional requirements for *p*AB and DHB were observed on defined medium, made with purified agar to minimize *p*AB contamination, and containing citrate, a known iron chelator, to reduce iron availability; DHB was not needed if the medium was supplemented with ferrous sulphate. Strains SL3217 and SL3218 were then tested for pathogenicity by intraperitoneal (i.p.) injection of bacteria (grown in nutrient broth with tetracycline at 5 μg ml^{-1}) into adult mice of the *Salmonella*-susceptible[9] inbred line C57BL. Mice given 3×10^5 live bacteria of either of the two *aroA554*::Tn*10* strains showed no ill effects; the LD_{50} for the *aro*$^+$ parent strain, SL3201, was <20 bacteria.

Strains auxotrophic because of Tn*10* insertion in a biosynthetic gene can regain their lost biosynthetic ability and lose tetracycline resistance by 'clean excision' or deletion of the transposon[5,6]; for strains SL3217 and SL3218 the frequency of reversion to aromatic independence (and tetracycline sensitivity) was $\sim10^{-8}$ per bacterium per generation. Such *aro*$^+$ revertants had regained virulence (LD_{50}<20, i.p.). To obtain non-reverting *aro*$^-$ strains we made use of the tendency of Tn*10* to cause DNA alterations within the transposon itself, sometimes extending into adjacent chromosomal genes. Many such deletion or deletion–inversion events cause both loss of tetracycline resistance and inability of the affected gene to revert to the wild-type form[10,11]. Tetracycline-sensitive variants of the *aroA554*::Tn*10* transductant SL3218 were found amongst the survivors in a culture exposed to ampicillin at 2,500 μg ml^{-1} while growing in the presence of tetracycline (5 μg ml^{-1}), a concentration bacteriostatic for cells lacking Tn*10*. A tetracycline-sensitive variant thus obtained, SL3235, did not revert to *aro*$^+$ at detectable frequency ($<10^{-11}$ per bacterium per generation). We also derived an *aroA554*::Tn*10* transductant, SL1346, from a genetically marked subline, SL1344, of *S. typhimurium* strain S2337; this strain differs from SL3201 both in biotype and in that it is virulent for calves[12]. A non-reverting, tetracycline-sensitive variant, SL3261, was isolated from strain SL1346 by plating on nutrient agar containing autoclaved chlortetracycline and fusaric acid, a combination which inhibits growth of tetracycline-resistant bacteria[13]. Both the stable *aroA*$^-$ strains, SL3235 and SL3261, were non-virulent for mice of another *Salmonella*-susceptible inbred line, BALB/c (ref 9) (no deaths from inocula of $\sim3\times10^6$ bacteria i.p., compared with LD_{50} of <20 for the *aro*$^+$ parent strains).

In addition to the requirement for aromatic amino acids, *p*AB and DHB, *aro* mutants will lack two other products of chorismate: ubiquinone, made via *p*-hydroxybenzoate, and menaquinone, made via *o*-succinylbenzoate. Lack of some chorismate-derived metabolite may account for two recently noted consequences of *aro* defect: failure to hypermodify a uridyl base in tRNA, reported by Björk[14] for *aro* mutants in *Escherichia coli*, and failure to produce H_2S from thiosulphate, noted by us for *S. typhimurium* with non-leaky blocks at *aroA* or *aroD*. We considered the possibility that some such consequence of *aro* defect might account, in part or entirely, for the non-virulence of *aro*$^-$ strains of *S. typhimurium*. However, a preliminary experiment showed that a large inoculum (2×10^8 bacteria i.p.) of the non-virulent *aro*$^-$ strain, SL3261, caused fatal infections in most BALB/c mice provided with drinking water containing both *p*AB and DHB; the same inoculum caused no apparent ill effects in mice which received *p*AB alone or DHB alone, or neither benzoate. This suggests that requirement for *p*AB and for DHB each by itself suffices to make strain SL3261 non-virulent for mice maintained on a normal diet, and that inability to synthesize other products of chorismate is not relevant to the non-virulence of *aro*$^-$ *S. typhimurium*.

Vaccination by injection of killed *Salmonella* confers only poor protection (reduction in mortality) against challenge with a virulent strain[9]. We therefore tested the ability of our stable, *aro*$^-$ strains, given i.p. as live vaccine, to protect against later challenge with a virulent *S. typhimurium* strain, given i.p. or by feeding (Table 1). The *S. typhimurium* strain used as challenge, SL1344 (the genetically marked virulent grandparent of *aro*$^-$ live vaccine strain SL3261), is virulent for mice, given i.p. or by feeding, and for calves by the oral route[12]. The mice used (the susceptible BALB/c line) were given graded i.p. inocula of one or the other stable *aro*$^-$ strain and were challenged 1 month later with the virulent strain, either 5×10^5 bacteria i.p. or 3×10^7 bacteria given by feeding (on bread cubes, after a 12-h fast). Mice challenged by feeding were housed one per cage to prevent cross-infection. Each of the two *aro*$^-$ strains, given i.p. in doses of 3×10^4, 3×10^5 or 3×10^6 (five mice per dose), gave complete protection against the oral challenge (no deaths and no apparent illness in 120 days of observation), whereas all five non-immunized control mice were dead by day 12. The two larger doses (2×10^5 and 2×10^6) of the *aro*$^-$ strain SL3261 protected all mice (five mice per dose) against i.p. challenge and the smallest dose used, 2×10^4 live bacteria, protected four of five mice (Table 1). Thus i.p. injection of as few as 2×10^5 live *aro*$^-$ bacteria into mice of a *Salmonella*-susceptible inbred line, BALB/c, gave complete protection against later i.p. injection of 5×10^5 virulent *S. typhimurium* (that is, >25,000 LD_{50}).

These results are in striking contrast to the failure of killed vaccines to protect such mice; Robson and Vas[9] found that six i.p. doses of killed *S. typhimurium* vaccine failed to protect BALB/c (or C57BL) mice against the lethal effect of i.p. injection of even as few as 10 cells of a virulent *S. typhimurium* strain. Oral administration of live bacteria of a non-virulent *galE* mutant of *S. typhi* has recently been shown to prevent typhoid fever in school children in Egypt[15]. Taken together, the data suggest that live vaccines may be useful for protecting man or domestic animals against *Salmonella* infections. Our results further suggest that strains with complete, non-reverting blocks in aromatic biosynthesis may be appropriate for such purposes.

This work was supported by US PHS grant AI07168 from the National Institute of Allergy and Infectious Diseases, by US Army Medical Research and Development Command contract DAMD-17-79-C-9076, and by a research contract with SmithKline Animal Health Products. S.K.H. was supported by National Research Service Award no. NIGMS 07276 and a NSF predoctoral fellowship. We thank Dr J. Roth of the University of Utah for the LT2 *aroA554*::Tn*10* insertion strain.

Table 1 Challenge by i.p. or oral administration of virulent *S. typhimurium* strain SL1344 (*aro*$^+$) to BALB/c mice immunized by i.p. injection of live *aro*$^-$ *S. typhimurium*

aro$^-$ live vaccine Strain	Dose	Challenge by *aro*$^+$ strain SL1344 Deaths/No. tested (days to death)
a Challenge by injection of 5×10^5 bacteria i.p. 34 days after live vaccine		
Controls	None	5/5 (day 2, 3, 3, 4, 5)
SL3261	2×10^4 i.p.	1/5 (day 3)*
	2×10^5 i.p.	0/5*
	2×10^6 i.p.	0/5*
b Challenge by feeding 3×10^7 bacteria 30 days after live vaccine		
Controls	None	5/5 (day 7, 8, 9, 9, 12)
SL3261	3×10^4 i.p.	0/5†
	3×10^5 i.p.	0/5†
	3×10^6 i.p.	0/5†
SL3235	3×10^4 i.p.	0/5†
	3×10^5 i.p.	0/5†
	3×10^6 i.p.	0/5†

Mice used were 15–17-week-old BALB/c males.
* All surviving mice were apparently well on day 60 after challenge.
† All mice alive and apparently well on day 120 after challenge.

Received 19 November 1980; accepted 2 March 1981.

1. Bacon, G. A., Burrows, T. W. & Yates, M. *Br. J. exp. Path.* **31**, 714–724 (1950).
2. Bacon, G. A., Burrows, T. W. & Yates, M. *Br. J. exp. Path.* **32**, 85–96 (1951).
3. Herzberg, M. *J. infect. Dis.* **111**, 192–203 (1962).
4. Yancey, R. J., Breeding, S. A. & Lankford, C. E. *Infect. Immun.* **24**, 174–180 (1979).
5. Kleckner, N., Chan, R. K., Tye, B. & Botstein, D. *J. molec. Biol.* **97**, 561–575 (1975).
6. Kleckner, N. *Cell* **11**, 11–23 (1977).
7. Schmieger, H. *Molec. gen. Genet.* **119**, 75–88 (1972).
8. MacPhee, D. G. & Stocker, B. A. D. *J. Bact.* **100**, 240–246 (1969).
9. Robson, H. G. & Vas, S. I. *J. infect. Dis.* **126**, 378–386 (1972).
10. Kleckner, N., Roth, J. & Botstein, D. *J. molec. Biol.* **116**, 125–159 (1977).
11. Kleckner, N., Reichardt, K. & Botstein, D. *J. molec. Biol.* **127**, 89–115 (1979).
12. Wray, C., & Sojka, W. J. *Res. vet. Sci.* **25**, 139–143 (1978).
13. Bochner, B. R., Huang, H., Schieven, G. & Ames, B. N. *J. Bact.* **143**, 926–933 (1980).
14. Björk, G. R. *J. molec. Biol.* **140**, 391–410 (1980).
15. Wahdan, M. H. *et al. Bull. Wld Hlth Org.* **58**, 469–474 (1980).

VOLUME XIV, NO. 114 | TUESDAY, MARCH 29, 1983 | BERKELEY, CALIFORNIA

Vaccine reaction

Student death termed accidental

By MAXINE WONG

After nearly three months of inquiry into the case of a UC Berkeley student who died shortly after receiving a typhoid vaccination at Cowell hospital, the San Francisco County coroner said yesterday the student succumbed to an "idiosyncratic reaction" to the vaccine.

According to Dr. Boyd Stephens, chief medical examiner for the coroner's office, an idiosyncratic reaction is "a personalized, severe type of response that's unusual and unpredictable."

In his report, Stephens labeled the mode of the student's death "accidental," although he said in an interview that this does not necessarily imply a lack of fault or responsibility for the death.

The 21-year-old student, whose name has been withheld to protect her family from publicity, died in a San Francisco hospital on Jan. 9, 1983, three days after receiving a typhoid vaccination at Cowell hospital. The vaccination was required for a medical microbiology and immunology course in which the student was enrolled at UC Berkeley.

The sudden death of "Grace," a senior who was double-majoring in biochemistry and microbiology, sparked concern and speculation among health officials because Grace was reportedly in good health before receiving the typhoid vaccination and because the official cause of her death was unknown for some time.

Stephens said an idiosyncratic reaction to the typhoid vaccine resulting in death is extremely uncommon. "I'm unaware of any other case reported," said Stephens.

Cowell hospital Assistant Administrator Virgie Tillman said the hospital had no comment about the coroner's findings. "We simply have nothing to add," Tillman said.

After Grace's unexplained death, the use of the typhoid vaccine at Cowell hospital was suspended for five days until it was determined by Cowell officials that the vaccine did not pose a threat to the campus community.

Dr. M.Z. Bierly of the Philadelphia headquarters of Wyeth Laboratories — the manufacturer of the typhoid vaccine — said he has received no other reports of idiosyncratic reactions caused by the vaccine.

"I've been here 30 years and no similar situation has been reported to me," Bierly said.

Stephens said that Grace's reaction to the vaccination caused her platelet count — the number of cell particles which aid the blood-clotting — to drop to an abnormally low level.

The sudden drop in her platelet count, in turn, caused Grace to spontaneously bleed to death, Stephens said.

Accidental

FROM FRONT PAGE

p 6.

400,000 platelets per cubic milliliter of blood. "People who are losing platelets rapidly can start bleeding when they get down to levels of around 50,000 (platelets per cubic milliliter of blood)," Stephens said.

Stephens said that Grace, at the time of her death, had a platelet count of only 8,000 platelets per cubic milliliter of blood.

"That puts her in the position where any type of trauma — even just the trauma of the normal pulse — can cause bleeding to start and once it starts, you can't stop it," Stephens said.

Stephens said there is no "practical" test to find out whether or not an individual is unusually sensitive to the typhoid vaccine.

"In theory, you can do something (to test for possible adverse reaction to typhoid vaccine), but as far as screening someone, there's no reasonable test that's available," Stephens said.

Stephens added that the lack of a "reasonable" test to determine possible adverse reaction to typhoid vaccine is due to the extremely low incidence of such reactions.

Bibliography of publications supported by contract.

Hoiseth, S. K., and Stocker, B. A. D. Aromatic-dependent Salmonella typhimurium are non-virulent and effective as live vaccines. 1981, Nature, Lond. 291, 238-293 (Included in this report as Appendix A).

Hoiseth, S.K. Aromatic-deficient mutants as live Salmonella vaccines. Stanford Ph.D. Thesis, 1982.

Personnel receiving contract support.

Dr. Bruce A. D. Stocker, Professor: part salary.

Dr. Robert J. Roantree, Associate Professor: part salary.

Susan K. Hoiseth, Graduate Student; Ph.D. degree December,1972; stipend from NSF but research expenses from contract.

Ronald F. Brown, Graduate Student; Thesis research completed but thesis not yet submitted.

Cheryll L. Barrett, Life Science Research Assistant; part salary.